**Acarological risk of exposure to agents of tick-borne zoonoses in the first recognized Italian focus of Lyme borreliosis**

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# Acarological risk of exposure to agents of tick-borne zoonoses in the first recognized Italian focus of Lyme borreliosis

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## SUMMARY

Acarological risk was calculated as the probability of encountering at least one host-seeking *Ixodes ricinus* tick infected by the pathogen *Borrelia burgdorferi sensu lato*, in 100 m transects in the province of Genoa, Italy. The seasonal pattern of *I. ricinus* was studied using generalized estimating equations (GEE) with negative binomial error, to consider overdispersion of tick counts and repeated sampling of the same dragging sites from April 1998 to March 1999. Prevalence of infection by *B. burgdorferi s.l.* was evaluated by PCR and hybridization with genospecies-specific probes. Acarological risk ($R$) peaked in April ($R=0{\cdot}2$, 95% CI 0·13–0·26) and November ($R=0{\cdot}29$, 95% CI 0·10–0·46). *Borrelia garinii* and *B. valaisiana* were the most common genospecies at our study site suggesting a major role of birds as reservoirs. DNA from *Anaplasma phagocytophilum*, the agent of granulocytic ehrlichiosis in humans and animals, was amplified from an adult *I. ricinus*.

## INTRODUCTION

The first Italian case of Lyme borreliosis, a tick-borne zoonosis caused by the spirochaete *Borrelia burgdorferi sensu lato* (*s.l.*) [1], was reported in 1984 from the village of Borzonasca, in the province of Genoa, in the northwestern region of Liguria [2]. The disease's annual incidence rate in the same area was estimated to be 17 human cases per 100 000 inhabitants [3]. In Italy, the intensity of transmission of *B. burgdorferi s.l.* by the tick vector *Ixodes ricinus* varies geographically, with the highest prevalences of spirochaetes in host-seeking ticks being reported from northeastern regions [4, 5]. *Anaplasma phagocytophilum*, the aetiological agent of human granulocytic ehrlichiosis (HGE), was identified in ticks in Italy in 1998 [6], and specific antibodies were detected in dogs from Liguria [7]. Under these circumstances, the risk of exposure to pathogen-infected *I. ricinus* for people and other susceptible animals (acarological risk) should be assessed in the Borzonasca area. Such a risk measure can be estimated as the mean number of infected, host- seeking ticks per 100 m² of ground [8].

*Borrelia burgdorferi s.l.* can be further classified in genospecies based upon genetic and phenotypic differences [1]. Three genospecies are presently known to cause the disease in Europe: *B. garinii* causes neuroborreliosis and is mostly associated with birds as reservoir hosts; whereas *B. afzelii* which causes cutaneous lesions, and *B. burgdorferi sensu stricto* (*s.s.*)

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which causes joint disease, are found in small and medium-sized mammals [9]. Therefore, geographical variations in the relative frequency of genospecies may be associated with ecological and clinical patterns of Lyme borreliosis.

In this study, we collected host-seeking *I. ricinus* in Liguria, and applied statistical models for overdispersed, correlated data to obtain valid estimates of tick abundance and seasonality [10]. We also carried out laboratory analyses to identify *B. burgdorferi s.l.*, as well as *A. phagocytophilum*, in *I. ricinus*, and characterized these agents by genetic techniques. Tick abundance data and laboratory results were integrated to obtain point estimates and confidence intervals (CIs) of an acarological risk index.

## METHODS

### Tick collection

Host-seeking ticks were collected during 11 monthly sessions, from April 1998 to March 1999, excluding February 1999. Six inland sites were selected, based on accessibility, which encircled the inhabited centre of the village of Borzonasca (2000 inhabitants, approximately 50 km east of Genoa, and 13 km from the sea). Three sites were in the immediate vicinity of housing and three within 1 km. The area is characterized by the presence of other villages of approximately the same size as Borzonasca, resulting in a population density of 114 people per km² [information provided by the Consortium of Municipalities (Comunità Montana) of Aveto, Graveglia, Sturla]. Five additional tick collection sites were within 2 km from the sea, on the northern slopes of hills surrounding the coastal town of Chiavari (27 500 inhabitants). Here, the high density of houses and enclosed private properties limited sampling, and four out of five sites were within 3 km of each other. All sites were on hills covered by deciduous woods, dominated by chestnuts (*Castanea sativa*), oaks (*Quercus cerris*), and black locust (*Robinia pseudacacia*).

At each site, ticks were collected by dragging on 50 m transects by two operators. One of them used a tick drag comprised of 12 flannel strips, the other a 1 m² cotton cloth. Ticks that were collected on a site by the two operators were pooled together and maintained alive in humidified vials or preserved in 70% ethanol and subsequently identified using keys from Manilla and Iori [11]. Data on rainfall and temperature in Borzonasca, from April 1998 to March 1999, were provided by the Hydrographic and Mareographic Office (Ufficio Idrografico e Maregrafico) of Genoa.

### Detection of *B. burgdorferi s.l.* in ticks

*Borrelia burgdorferi s.l.* isolation was attempted from 10 adult and 18 nymphal *I. ricinus* from Borzonasca and from 10 adult and 12 nymphs from Chiavari, in pools of 4–10, as previously described [12]. For the characterization of isolates by genospecies, restriction fragment length polymorphism (RFLP) was employed as described by Postic et al. [13].

For pathogen detection by PCR, host-seeking *I. ricinus* (115 nymphs and 55 adults) were individually homogenized with a pestle in microcentrifuge tubes. After extraction by Isoquick (Orca Research, Bothwell, WA, USA), DNA was suspended in 50 $\mu$l of nuclease-free water and utilized (5 $\mu$l per reaction) in a nested PCR as described by Rijpkema et al. [14], using primer pairs specific for the intergenic spacer region comprised between genes codifying for subunits 5S and 23S of ribosomal RNA. The variability of the amplified fragment allows characterization of *B. burgdorferi s.l.* species. The cycling profile described in the original paper was used [14]. As positive controls, we alternatively used DNA from a *B. garinii* strain isolated within this study, *B. burgdorferi s.s.* (Alcaide strain), *B. afzelii* (Nancy strain), *B. valaisiana* VS 116 strain. Distilled water and *I. scapularis* nymphs from a colony maintained at the Yale School of Medicine (kindly provided by Professor D. Fish), and DNA extracted from bacteria (*Escherichia coli*, *Staphylococcus intermedius*, *Streptococcus* spp.) were used as negative controls. Amplified DNA underwent electrophoresis on agarose gel, and was visualized in ethidium bromide.

### Hybridization with DNA probes

In order to characterize amplified DNA by species, we carried out hybridization with oligonucleotide probes designed by Rijpkema et al. [14]. One probe was specific for *B. burgdorferi s.l.*, whereas the remaining four were specific for *B. burgdorferi s.s.*, *B. garinii*, *B. afzelii* and *B. valaisiana*. Probes were 3′-tailed, labelled with digoxigenin-11-dUTP and quantified. Amplicons were denatured at 95 °C for 5 min with 0·1 vol. of 4 M NaOH, 0·1 M EDTA, spotted (2 $\mu$l/spot) on a positively charged nylon membrane and cross-linked at 120 °C for 30 min. Prehybridization was carried out for 1 h at 55 °C while

the hybridization solution, containing 10 pmol/ml of each labelled oligoprobe was allowed to react overnight at the same temperature. After post-hybridization washes and immunostaing procedures, positive spots were identified by colorimetric detection. All procedures were carried out according to the digoxigenin system user's guide (Boehringer–Mannheim, Germany). Amplicons of positive controls for each *B. burgdorferi s.l.* genospecies that were included on each membrane allowed checking for cross reactions.

### Detection of *A. phagocytophilum* in host-seeking ticks

DNA from 19 *I. ricinus* adults and 41 nymphs was used in PCR using primers EHR521 and EHR747 that amplify a 247-bp fragment of 16S rDNA [15]. Positive controls were pools of *I. scapularis* nymphs that were fed of *Peromyscus leucopus* mice infected by the agent of HGE, from a colony maintained at the Yale School of Medicine (kindly provided by Professor D. Fish). The amplification products were visualized in 2·5% agar gel. If positive by electrophoresis, samples were subsequently sequenced.

### DNA sequencing

Amplicons were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Sequencing of PCR products (both strands) were performed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) by the dideoxy-chain termination method with fluorescent dye terminators, using PCR-derived primers. Sequences were analysed by the software CHROMAS 2.0 (Technelysium, Helensvale, Australia), and submitted to BLAST [16]. The determined sequences were aligned with the corresponding sequences of *A. phagocytophilum* strains by using the multisequence alignment program ClustalX [17].

### Statistical analysis

Transects of 100 m, the sum of 50 m per operator per site, were the units of dragging data analysis. The proportions of tick-positive transects, where at least one host-seeking tick was collected, were calculated for dragging session and tick stage. Exact binomial 95% CIs were obtained by the Fisher exact method using the Epi-Info software [18]. To obtain estimates of mean numbers of host-seeking ticks per transect and 95% CIs, we applied intercept-only, generalized log-linear models using the GENMOD procedure in the SAS® system (Exp option in the ESTIMATE statement, SAS version 8.2 [19]). Negative binomial error was used in the statistical models in order to take into account the potential overdispersion of the distribution of host-seeking ticks among dragging sites.

To estimate tick seasonal patterns and the effect of proximity to the sea (inland *vs.* coastal sites) on the numbers of collected ticks, we used generalized estimating equations (GEE) for counted responses with negative binomial error for larvae, nymphs and adult *I. ricinus* (PROC GENMOD) [19]. Using GEE (exchangeable correlation structure), we accounted for correlation arising from repeatedly collecting ticks at the same sites across the study period [20].

Seasonal pattern of larvae was modelled by sinusoidal fluctuations with an amplitude of 1 and a period of 6 months, with peaks in January and July, whereas sinusoidal functions with one peak in April, or with two peaks in April and October, were fitted for nymphs and adults, respectively. Distance from the sea was coded as COAST = 1 for coastal sites and COAST = 0 for inland sites. The inclusion of location × time interaction allowed us to test differences in time pattern between inland and coastal sites. The fit of the models and the presence of outliers were investigated by plotting Pearson residuals against predicted values.

Proportions of ticks that were PCR-positive for *B. burgdorferi s.l.* and *A. phagocytophilum* were calculated for each stage. Mean numbers of host-seeking nymphs and adults per transect were combined with the prevalence of infection by pathogenic *B. burgdorferi s.l.* species (*B. burgdorferi s.s.*, *B. afzelii*, *B. garinii*) to obtain monthly estimates of the acarological risk ($R$), the probability of collecting at least one infected tick in a 100 m transect. In the following equation:

$$R = 1 - (e^{-(\mu_{in} + \mu_{ia})}), \quad (1)$$

estimates of the mean numbers of infected nymphs ($\mu_{in}$) and adults ($\mu_{ia}$) per 100 m transect were obtained as the products of the mean numbers of ticks (of each stage) and observed infection prevalences. Confidence intervals of $R$ were obtained, for each month, by a bootstrap method, that consisted in sampling with replacement 11 observations from the original set of the 11 transects, for 100 times. The SAS System macro facility was used to generate statements and commands for multiple simulated data sets [21].

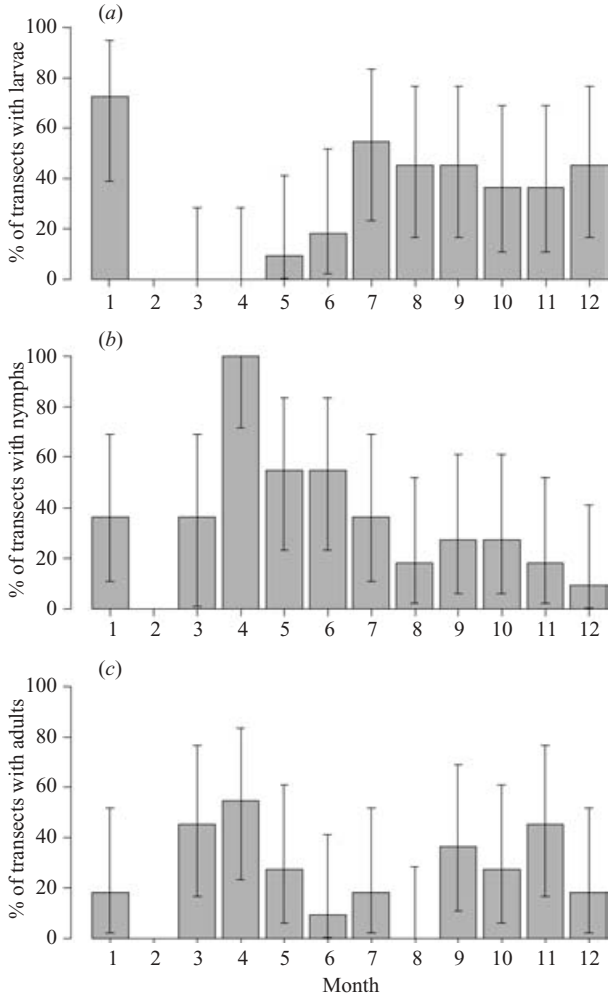


**Fig. 1.** Percentages and 95% CIs, of transects where at least one host-seeking *I. ricinus* was collected by dragging, from April 1998 to March 1999, in the province of Genoa: (*a*) larvae; (*b*) nymphs; (*c*) adults. For a better representation of seasonal pattern, months are reported from January 1999 to December 1998, although sampling started in April 1998 (4).

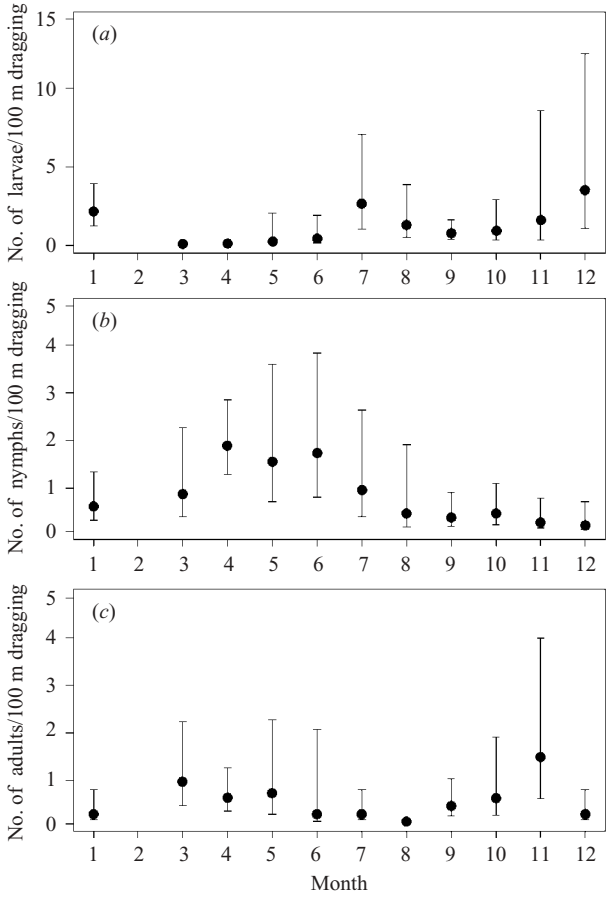


**Fig. 2.** Mean numbers and 95% CIs, of host-seeking *I. ricinus* that were collected by dragging in 11 transects in the province of Genoa, from April 1998 to March 1999: (*a*) larvae; (*b*) nymphs; (*c*) adults.

Simulated numbers of *I. ricinus* nymphs and adults were used to calculate *R*. The 5th and the 95th percentiles of the distributions of *R* were then chosen as the lower and upper limits of 95% CIs.

## RESULTS

During dragging sessions from April 1998 to March 1999, we collected 257 host-seeking *I. ricinus* larvae, 96 nymphs, and 57 adults. Three *Dermacentor marginatus* adults were collected in March 1999. Larvae were found in 40 out of 121 (33·1%) dragging observations (11 transects repeatedly sampled during 11 sessions), nymphs were collected in 46 observations (38·0%), and adults in 33 observations (27·3%). Larval *I. ricinus* showed a summer peak of activity in July (2·7 larvae per transect, 95% CI 1·0–7·4), they were subsequently found during autumn and winter, and peaked again in December (3·6 larvae per transect, 95% CI 1·0–12·8) (Figs 1*a* and 2*a*). In January, larvae were found across most of the study area (at least one larva in 8 out of 11 transects; Fig. 1*a*); in fact, during this month, the 95% CI of the mean number of larvae per transect (2·2) was relatively narrow (1·2–4·0, Fig. 2*a*). At coastal sites, larval activity decreased in autumn before rising again in winter, whereas at inland sites such a decrease was not observed (results not shown). Such differences yielded a significant location × time interaction in the log-linear regression ($P<0·005$, Table 1). Statistical analyses on larval counts were carried out after excluding an outlier observation of 109 larvae that were collected in November at an inland transect, and probably originated during the hatching of

Table 1. *Results of log-linear regression (fitted using GEE with negative binomial error and exchangeable correlation) of counts of host-seeking* I. ricinus *ticks in 11 transects in Liguria, from April 1998 to March 1999*

| | Parameter estimates (S.E.) | | | | | | |
|---|---|---|---|---|---|---|---|
| Stage | Intercept | Seasonal SINE | COAST | INTER | $\rho$ | $k$ (95% CI) | Deviance (D.F.) |
| Larvae | 0·41 (0·43) | 0·72 (0·25)<br>$P<0·005$ | −1·3 (0·72)<br>$P=0·07$ | 1·2 (0·39)<br>$P<0·005$ | 0·13 | 0·28 (0·18–0·46) | 83·4 (117) |
| Nymphs | −0·35 (0·31) | 1·0 (0·13)<br>$P<0·001$ | −0·21 (0·49)<br>$P=0·66$ | — | 0·17 | 1·0 (0·48–2·1) | 104·6 (118) |
| Adults | −1·3 (0·21) | 0·91 (0·21)<br>$P<0·001$ | 0·54 (0·44)<br>$P=0·22$ | — | 0·03 | 0·63 (0·27–1·4) | 83·3 (118) |

INTER, time × location interaction; $\rho$, correlation coefficient; $k$, negative binomial parameter.

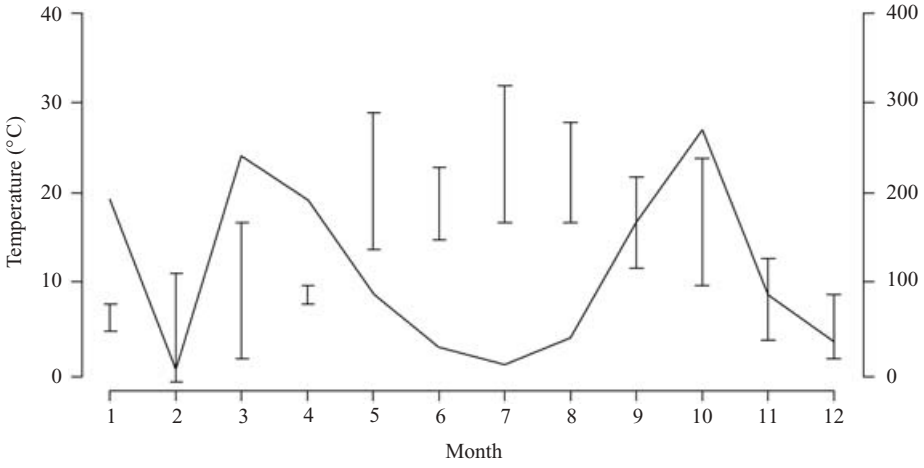


**Fig. 3.** Monthly rainfall (line) and minimum and maximum temperatures (vertical bars) in the village of Borzonasca (province of Genoa) from April 1998 to March 1999.

a cluster of eggs from one female tick. Nymphs were collected during all sessions but were most abundant during spring. A significant peak of nymph activity was observed in April (mean = 1·9, 95% CI 1·2–2·9, $P<0·001$; Figs 1*b* and 2*b*, Table 1) when all transects were positive for this stage. Adult *I. ricinus* were active in spring and, more markedly, in autumn: 0·90 (0·37–2·3) adults per transect were collected in March and 1·5 (0·51–4·1) in November ($P<0·001$; Figs 1*c* and 2*c*, Table 1). Seasonal patterns of numbers of nymphs and adults did not differ between inland and coastal sites (Table 1), and SINE × COAST interactions were therefore not included in the statistical models. The negative binomial parameter was smallest for larvae ($k=0·28$, 95% CI 0·18–0·46), indicating aggregated distribution of this stage. In Borzonasca, rainfall peaked in March and October. July was characterized by the lowest rainfall and highest temperature. Minimum temperature decreased below zero only in February (Fig. 3).

### Infection of *I. ricinus* with *B. burgdorferi s.l.*

Two *B. burgdorferi s.l.* strains were isolated from adult *I. ricinus* collected at Borzonasca and one strain was isolated from adults from Chiavari. The three isolates were subsequently characterized as *B. garinii* by PCR–RFLP (Fig. 4). Thirty-one out of 170 (18·2%) host-seeking *I. ricinus* that were tested by PCR for *B. burgdorferi s.l.* resulted positive, as determined by hybridization of amplified DNA with a group-specific probe. Prevalence of infection was significantly greater in adults (21/55, 38·2%) than in nymphs (10/115, 8·7%, $\chi^2=19·8$, 1 D.F., $P<0·001$). *Borrelia garinii* was the most frequent genospecies that was identified by species-specific probes (Table 2), whereas prevalence of *B. valaisiana* was the same as prevalence of *B. garinii* in adults but lower in nymphs. *Borrelia afzelii* constituted approximately 10% of PCR + specimens, whereas *B. burgdorferi s.s.* was not identified in our sample. One adult *I. ricinus*

Table 2. *Identification and characterization of* B. burgdorferi s.l. *by PCR and hybridization with* B. burgdorferi s.l.*-specific, and genospecies-specific probes, in host-seeking* I. ricinus *in 11 transects in Liguria, from April 1998 to March 1999*

| | Genospecies [no. positive (%)] | | | | | |
|---|---|---|---|---|---|---|
| Stage | *B. burgdorferi s.l.* | *B. garinii* | *B. afzelii* | *B. burgdorferi s.s.* | *B. valaisiana* | n.d. |
| Nymphs ($n=115$) | 10 (8·7) | 4 (3·5)* (40·0)† | 1 (0·87) (10·0) | 0 (0·0) (0·0) | 1 (0·87) (10·0) | 4 |
| Adults ($n=55$) | 21 (38·2) | 9 (16·4) (42·8) | 2 (3·6) (9·5) | 0 (0·0) (0·0) | 9 (16·4) (42·8) | 1 |
| Total ($n=170$) | 31 (18·2) | 13 (7·6) (41·9) | 3 (1·8) (9·7) | 0 (0·0) (0·0) | 10 (5·9) (32·2) | 5 |

* Percentage of all ticks of a certain stage that were infected with a genospecies.
† Percentage of positive specimens that were infected with a genospecies.
n.d., Not determined.

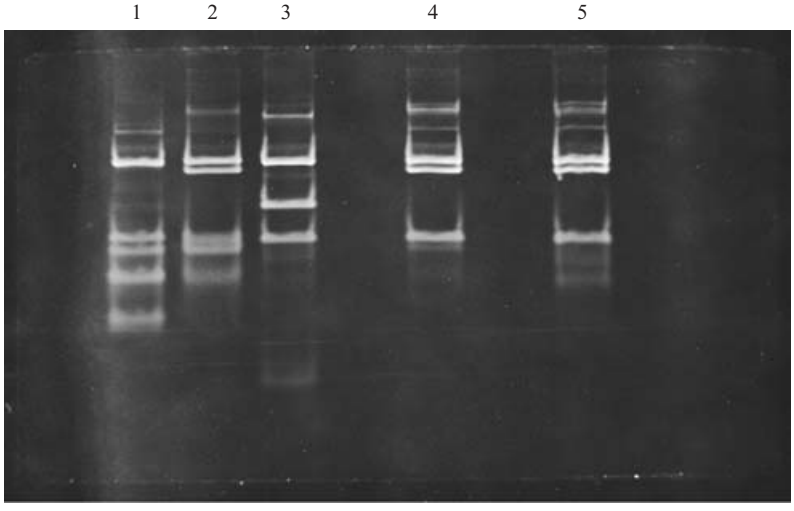


**Fig. 4.** Restriction patterns of *B. burgdorferi s.l.* strains isolated from adult *I. ricinus* ticks in the province of Genoa. Lane 1, *B. burgdorferi s.s.*; lane 2, *B. garinii*; lane 3, *B. afzelii*; lane 4, Chiavari strain; lane 5, Borzonasca strain. Strains in lanes 4–5 were classified as *B. garinii*.

was co-infected by *B. garinii* and *B. valaisiana*, and another adult by *B. garinii* and *B. afzelii*.

*R*, the risk of finding at least one tick (adult or nymph) infected by a pathogenic *B. burgdorferi s.l.* species, was characterized by a bimodal seasonal pattern and peaked during the wettest seasons of spring and autumn (Figs 3 and 5). Although punctual *R* estimate was highest in November ($R=0{\cdot}29$, 95% CI 0·10–0·46), April was characterized by a more homogeneous risk across the study area, as shown by a narrower 95% CI ($R=0{\cdot}20$, 95% CI 0·13–0·26).

One adult tick from Chiavari was positive by PCR for *A. phagocytophilum* (Fig. 6). Subsequent sequence analysis confirmed such a finding, with 100% homology with reference sequences. Lack of variability of the amplified fragment prevented further phylogenetic analysis.

## DISCUSSION

At our study area, the seasonal pattern of host-seeking *I. ricinus* was characterized by a bimodal larval activity, with summer and late autumn–winter peaks. The mild climate of Liguria (Fig. 3) favoured the activity of larvae during autumn and winter. Conversely, we did not observe the larval spring peak reported at other locations [22], and no larvae

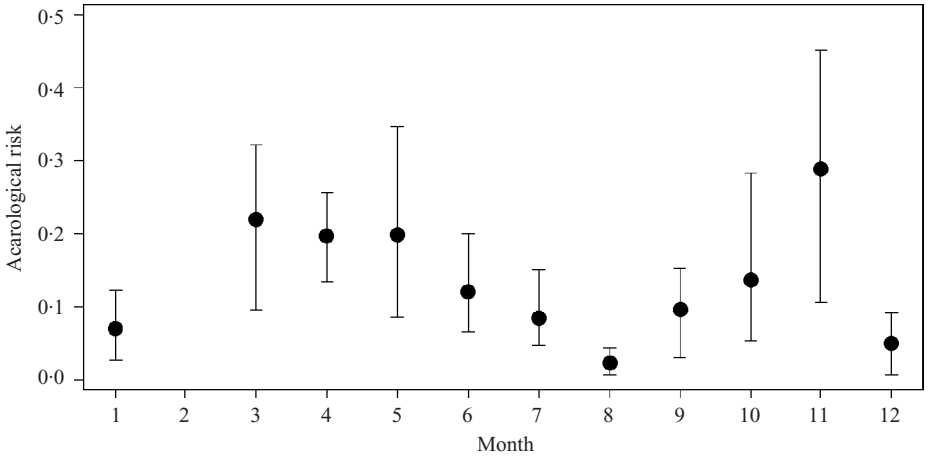


**Fig. 5.** Acarological risk (*R*), the probability of finding at least one *I. ricinus* tick (nymph or adult) infected with pathogenic *Borrelia burgdorferi s.l.* genospecies, in 11 transects in the province of Genoa, from April 1998 to March 1999. Vertical bars represent 95% CIs.

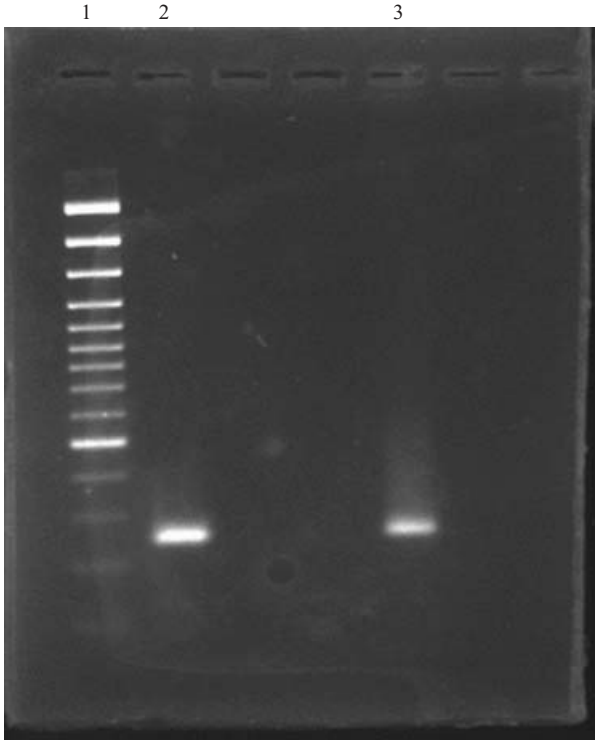


**Fig. 6.** Agarose gel electrophoresis of PCR product obtained by amplification, with primers specific for *A. phagocytophilum*, of DNA extracted from an adult *I. ricinus* from Chiavari, Genoa. Lane 1, 100-bp DNA ladder; lane 2, positive control; lane 3, 247-bp PCR-positive sample.

were collected during March and April. Nymphs were active during most of the year, with a significant spring peak. Numbers of questing nymphs/100 m dragging in Liguria (1·9 in April) were lower than those reported from the province of Trento (north-eastern Italy), where Rizzoli et al. [5] found up to 37·5 nymphs/100 m. Such differences are probably due to variable population densities of favourite hosts for adult ticks, such as the roe deer (*Capreolus capreolus*), which reaches 32 heads/100 ha in Trento but is absent from our study area. Adult *I. ricinus* were mostly active in the wettest months of spring and autumn and were not found in summer, and this pattern is similar to those observed at other Italian locations [4].

Our findings showed that larvae were the only tick stage characterized by an aggregated distribution of counts even after the inclusion of seasonal sine and proximity to the sea in the statistical models (Table 1). Hatching of clumps of eggs from individual female ticks and scarce lateral movements of larvae might lead to spatial aggregation [23].

A relatively low abundance of questing *I. ricinus* in Liguria might explain the correspondingly moderate intensity of *B. burgdorferi s.l.* transmission that we observed. In fact, 8·7% ($n=115$, Table 2) prevalence of infection in nymphs in Liguria was lower than the prevalence found in Trentino by Rizzoli et al. [5], and by Altobelli et al. [24] in the Karst of Trieste (up to 38% prevalence). Other European studies showed that prevalence of infection in questing nymphs is positively associated with tick abundance which, in turn, depends on deer population density. It is only above a relatively high threshold of deer density that such species' inability to serve as a reservoir for spirochaetes leads to a reduction of infection levels in questing ticks [25].

*Borrelia garinii* and *B. valaisiana*, the most frequent genospecies that we detected in host-seeking nymphs

and adults (Table 2), are mostly associated with birds [9], and avian species might be major reservoirs of spirochaetes in Liguria. However, recent studies showed that *B. garinii* serotype 4 was identified in wild rodents (D. Huegli et al., unpublished observations). Laboratory techniques that we used did not allow characterization of *B. burgdorferi s.l.* by serotype, and further analysis would be necessary. Higher infection levels in adults than in nymphs (38·2 *vs.* 8·7%) were largely attributable to *B. valaisiana* (Table 2). This finding might be explained by the particular competence of birds for nymphal *I. ricinus* [26]. The relative frequency of genospecies that we found in Liguria differs from findings by Cinco et al. [27] in the Friuli region where all four genospecies were found, frequently in association but with different rates. On the other hand, Ciceroni et al. [28] most commonly isolated *B. afzelii* from human patients in the Veneto region. Overall, our findings are similar to those reported from other European countries where *B. garinii* is the most common genospecies [9].

The risk of collecting at least one pathogenic *B. burgdorferi*-infected *I. ricinus* tick, as expressed by *R*, followed the seasonal pattern of nymphal and adult stages, with spring and autumn peaks. Nymphs are more difficult to detect than adults when attached to the skin, and they are therefore most likely to transmit the agents of Lyme borreliosis. Consequently, spring – when nymphs were most abundant – can be considered as the period with the highest risk of infection in Liguria. Moreover, the CI of *R* was narrower in April than in November, showing a more homogeneous distribution of risk of infection across the study area in spring.

The identification of *A. phagocytophilum* DNA in one host-seeking *I. ricinus* confirms a potential risk of HGE in the study area, as previously suggested by the detection of specific antibodies in dogs [7]. Although transmission of *A. phagocytophilum* generally occurs at low levels compared to *B. burgdorferi s.l.* [29], the risk of infection by such a zoonotic agent in Liguria should be considered in human and veterinary medicine.

## CONCLUSIONS

In the Borzonasca–Chiavari area, pathogen-infected *I. ricinus* were active during most of the year in the immediate vicinity of housing, indicating residential risk of tick-borne zoonoses. People can also be exposed to tick bites during leisure activities taking place in the woods in spring, and during mushroom collection and hunting in autumn. In summer, when large numbers of tourists are present on the coast of Liguria and often visit inland sites, acarological risk was relatively low (Fig. 4). Information on spatial and temporal distribution of acarological risk is important in preventive medicine, and should be obtained through locally targeted field studies in Italy, where climatic and environmental determinants of tick abundance and activity (such as temperature and humidity) undergo major changes within short distances.

## ACKNOWLEDGEMENTS

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